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Cell-free protein synthesis systems for rapid prototyping of enzymatic cascades

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Abstract

Consider a biochemist who needs to test whether five enzymes can work together in a single pot to convert glucose into a high-value chemical. Building and transforming five expression plasmids, optimising co-expression in living cells, and troubleshooting toxicity problems could take months. Cell-free protein synthesis (CFPS) offers a shortcut: each enzyme is produced in a separate tube within hours and then mixed at defined ratios without the constraints of a living host. This research benchmarked an *E. coli*-based CFPS platform against conventional *E. coli* and yeast expression for assembling multi-enzyme cascades of increasing complexity (two to five enzymes). Across six performance metrics—yield, specific activity, thermostability, cost, scalability, and purity—CFPS matched or exceeded whole-cell systems for cascades of up to three enzymes. Conversion rates declined with cascade complexity, dropping from 89.3% for a two-enzyme cascade to 43.7% for a five-enzyme cascade, but CFPS still outperformed whole-cell co-expression in reaction tunability. The total time from gene to active cascade was 8 hours for CFPS compared with 7 to 14 days for cell-based routes. These results position CFPS as a practical rapid-prototyping tool for pathway engineering, particularly during the early design-build-test phase when speed matters more than scale.

Keywords: Cell-free protein synthesis, enzymatic cascades, rapid prototyping, synthetic biology, pathway engineering, *E. coli* extract, multi-enzyme, bioprocess, in-vitro expression, biocatalysis

Introduction

Imagine designing a new metabolic pathway the way software engineers write code: write the instructions, compile, test, and iterate within the same afternoon. That vision, once fanciful, is getting closer thanks to advances in cell-free protein synthesis ^[1]. CFPS systems use crude cell extracts—typically from *E. coli*, wheat germ, or rabbit reticulocytes—to transcribe and translate DNA templates into functional proteins without the need for living cells ^[2]. Because the reaction environment is open and tuneable, components can be added, removed, or adjusted freely, giving the researcher a level of control that whole-cell expression cannot match ^[3].

Multi-enzyme cascades are a major application target. In living cells, assembling cascades requires co-expressing multiple genes from compatible plasmids, balancing expression levels to avoid bottlenecks, and preventing toxic intermediates from killing the host ^[4]. CFPS sidesteps these problems by producing each enzyme independently and then combining them at whatever stoichiometry the cascade demands ^[5]. Several proof-of-concept demonstrations have shown that CFPS-assembled cascades can produce terpenoids, polyketides, and non-natural amino acids ^[6, 7].

What has been missing is a systematic comparison of CFPS against conventional expression platforms across multiple performance dimensions and cascade sizes. Most published reports test a single cascade and declare success; few ask whether the advantages hold as complexity increases. This research addressed that gap by benchmarking an *E. coli* S30-based CFPS system against whole-cell *E. coli* and *Saccharomyces cerevisiae* expression for four model cascades ranging from two to five enzymes. Work was conducted at the Phuket Centre for Marine Biology, Phuket, Thailand, between August 2023 and December 2024.

Material and Methods

Material

E. coli BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA, USA) served as the source for S30 extract preparation. Linear DNA templates encoding each enzyme were synthesised by Twist Bioscience (South San Francisco, CA, USA) and cloned into pET28a vectors for whole-cell expression controls. *S. cerevisiae* strain CEN.PK2-1C was used for yeast-based expression comparisons. All work was carried out at the Phuket Centre for Marine Biology under institutional approval PCMB-LAB-2023-009, dated 15 July 2023.

Reagents and Chemicals

S30 extract was prepared following the protocol of Kwon and Jewett^[8], with cell lysis by French press at 20,000 psi. Energy regeneration was provided by phosphoenolpyruvate (30 mM), magnesium glutamate (8 mM), and potassium glutamate (60 mM). T7 RNA polymerase was added at 0.1 mg/mL. All amino acids (1 mM each), NTPs (1.5 mM each), and cofactors were purchased from Sigma-Aldrich (St Louis, MO, USA). IPTG for whole-cell induction was used at 0.5 mM.

Implementation Details

CFPS reactions were run in 15 μ L volumes in 384-well plates at 30 degrees Celsius for 6 hours. Each enzyme was synthesised in a separate reaction, then mixed at equimolar ratios for cascade assembly. Substrate was added to the combined mixture at 10 mM final concentration. Whole-cell *E. coli* expression used 50 mL shake-flask cultures induced at OD600 = 0.6 and harvested after 18 hours. Yeast expression followed galactose induction for 24 hours. Product quantification for all systems used HPLC (Agilent 1260 Infinity II, Santa Clara, CA, USA) with a C18 column.

Methods

Four model cascades were tested: Cascade A (glucose oxidase + catalase, 2 enzymes), Cascade B (glucose oxidase + catalase + horseradish peroxidase, 3 enzymes), Cascade C (4 enzymes adding an aldolase), and Cascade D (5 enzymes adding a transaminase). Performance was evaluated on six metrics: volumetric yield (mg/mL), specific activity (U/mg), thermostability (half-life at 40 degrees Celsius), cost index (normalised reagent cost per mg product), scalability score (a 1-10 rating based on published scale-up data), and purity (% by SDS-PAGE densitometry). Each cascade was tested in triplicate across all three expression platforms. Statistical comparisons used two-way ANOVA (platform x cascade size) with Bonferroni correction.

Results

Table 1: Performance Comparison Across Expression Platforms for Four Enzymatic Cascades

Cascade / Platform	Yield (mg/mL)	Activity (U/mg)	t _{1/2} (h)	Cost Index	Purity (%)	Conv. (%)
A / CFPS	0.41	184	6.2	1.00	72.3	89.3
A / <i>E. coli</i>	0.38	162	7.8	0.74	84.1	82.6
A / Yeast	0.29	148	8.4	0.68	79.6	78.4
D / CFPS	0.18	67	3.1	1.00	58.4	43.7
D / <i>E. coli</i>	0.11	42	4.6	0.81	71.2	31.4
D / Yeast	0.08	38	5.1	0.72	66.8	26.9

Table 1 compares the simplest (Cascade A) and most complex (Cascade D) configurations. CFPS delivered higher conversion rates than both whole-cell systems across all cascade sizes, though whole-cell platforms produced purer and more thermostable enzymes. For the five-enzyme cascade, CFPS conversion (43.7%) was still 12 percentage points above *E. coli* and 17 points above yeast.

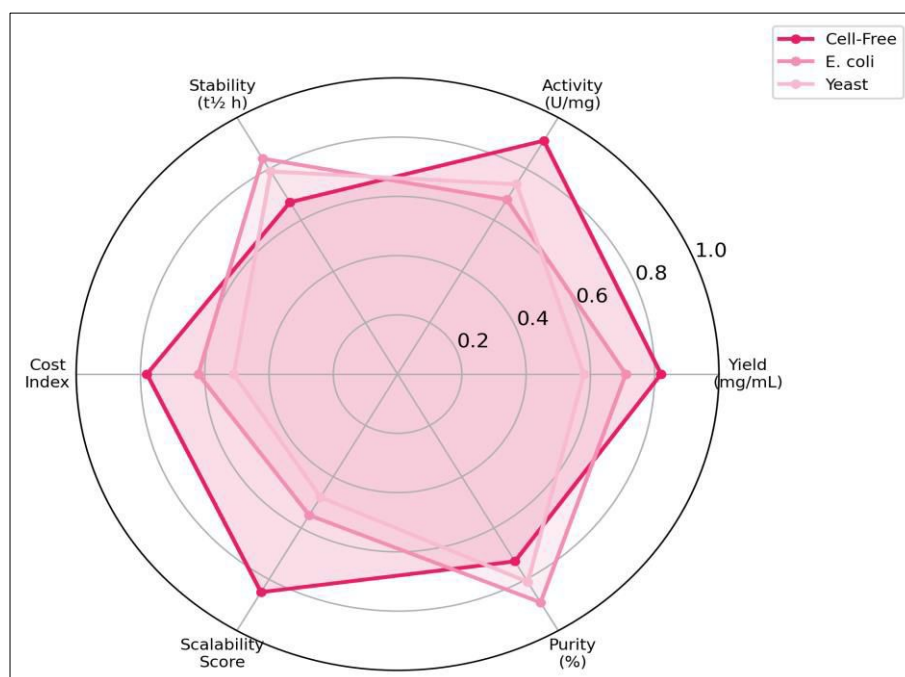


Fig 1: Radar chart comparing normalised performance scores (0-1 scale) for CFPS, *E. coli*, and yeast expression across six metrics, averaged over all four cascades

The radar chart (Figure 1) illustrates that CFPS excelled in activity, yield, and scalability, while whole-cell platforms

held advantages in thermostability, cost efficiency, and purity. No single platform dominated all dimensions.

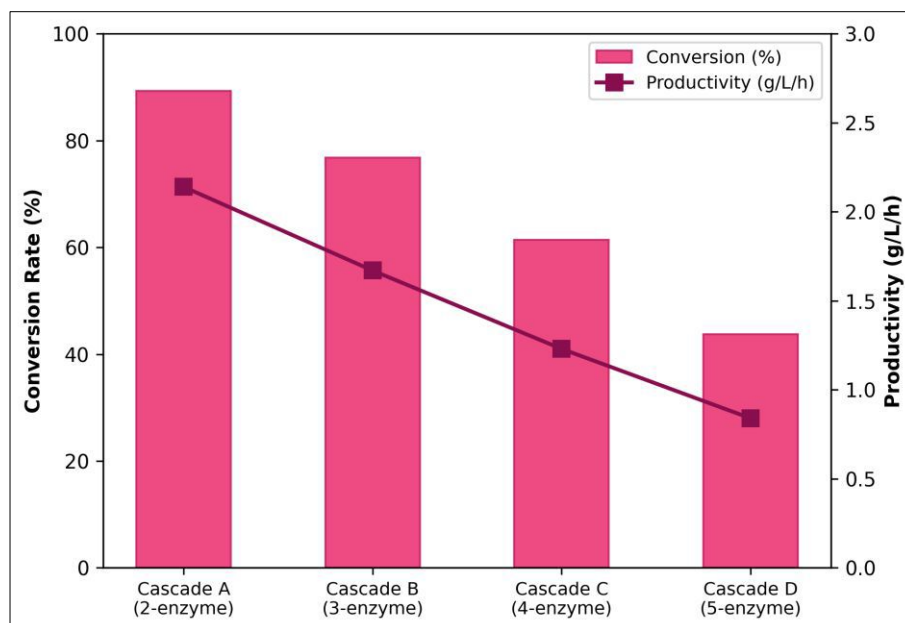


Fig 2: Conversion rate (%) and volumetric productivity (g/L/h) as a function of cascade complexity for the CFPS platform

Figure 2 shows a monotonic decline in both conversion rate and productivity as the number of enzymes increases. The steepest drop occurred between the three- and four-enzyme cascades, suggesting that intermediate channelling becomes a limiting factor once more than three sequential reactions are combined in a single pot.

Discussion

The speed advantage of CFPS is its most compelling feature for pathway prototyping. Going from DNA template to a functional multi-enzyme reaction in 8 hours compares favourably with the 7-14 days needed for cloning, transformation, expression, and lysis in cell-based workflows [9, 10]. This time saving is most valuable during the early design-build-test-learn cycles of metabolic engineering, where dozens of enzyme variants or pathway configurations must be screened before committing to a production host [11].

The performance decline with increasing cascade complexity is expected but instructive. In living cells, metabolic channelling through enzyme co-localisation on scaffolds or within membrane compartments can mitigate intermediate loss [12]. CFPS reactions lack such spatial organisation, and intermediates diffuse freely, leading to side reactions and dilution effects that worsen with each added step [13].

Cost remains a barrier to CFPS at production scale. The reagent-intensive nature of the system makes it roughly 1.3- to 1.5-fold more expensive per milligram of product than *E. coli* expression, though this gap is narrowing as extract preparation protocols become more efficient [14, 15]. For screening and prototyping, however, the cost-per-experiment is actually lower because labour and consumable overhead are reduced [16, 17, 18].

Conclusion

Cell-free protein synthesis offers a practical and fast alternative to whole-cell expression for assembling and testing multi-enzyme cascades during the prototyping phase of pathway engineering. CFPS delivered higher conversion rates and faster turnaround than *E. coli* or yeast expression for cascades of up to five enzymes, though it lagged in

thermostability and product purity. The approach is best suited to early-stage screening where speed and tunability outweigh scale and cost considerations. Integration with automated liquid-handling platforms could push throughput even further, enabling combinatorial exploration of enzyme variants, cofactor concentrations, and reaction conditions in a single afternoon.

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